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The Role of *Aerobacter* sp., *Escherichia coli* and Certain Amino Acids in the Excystment of *Schizopyrenus russelli*

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SUMMARY: Aqueous extracts of *Aerobacter* sp. and *Escherichia coli* have been found to cause excystment of viable sterile cysts of *Schizopyrenus russelli*. The factors which cause excystment are thermostable. With the aid of paper partition chromatography of the aqueous extract of *Aerobacter* sp., it has been found that part of the excystment-inducing activity is due to the presence of amino acids, some of which have been identified. Amino acids, sugars, purines, pyrimidines, nucleosides, nucleotides and organic phosphates have been tested for their ability to induce excystment. It has been found that some amino acids and a few nucleotides can cause excystment. The effect of pH, concentration and time on excystment with amino acids has been studied.

Bacteria and amoebae live in close association in human and animal intestine, and living bacteria in addition to being the usual food for ameobae are believed to be in some way responsible for the excystment of amoeboid cysts. A study of the amoeba excystment factor or factors produced by common intestinal bacteria might be of significance for more effective chemotherapy of chronic amoebic dysentery. Various factors such as hypo- and hypertonicity of the medium, low oxygen tension, change of pH value, presence of reducing agents or nutrient media, change of temperature, desiccation, presence of bacteria, etc., have been claimed to potentiate the emergence of trophozoites from their resting cysts in Protozoa (see Beers, 1946; Crump, 1950; Hall, 1953; Kudo, 1954; Singh, 1941*b* for the earlier literature). Earlier workers in this field, however, do not appear to have fully recognized the risk of contamination by other organisms inherent in such experiments, especially when nutritionally rich media are to be examined for the presence of excystment factors. Unless aseptic conditions are maintained during experiments, the results will remain open to doubt.

It was shown by Singh (1941*b*) for *Colpoda steinii*, by Beers (1946) for *Didinium nasutum* and by Crump (1950) for Amoeba 4(*Schizopyrenus russelli*, Singh, 1952) that an environment containing bacteria was necessary for the resting cysts of these Protozoa to excyst. Singh, Mathew & Sreenivasaya (1956) demonstrated that a strain of an *Aerobacter* sp. produced in an actively proliferating culture an excystment factor for *S. russelli* cysts, which was capable of diffusing easily through a collodion membrane; the factor was thermolabile and its activity was somewhat dependent on the pH value of the medium.

The present paper deals with the extraction and isolation of material causing excystment from an *Aerobacter* sp. and from *Escherichia coli* and examines the role of certain amino acids in excystment.

METHODS

Preparation of sterile cysts of Schizopyrenus russelli

The cysts from a 'pure line' culture of amoebae were used in this work (see Singh, 1952, for the characters of *Schizopyrenus russelli* and the culture methods used). The cysts are double-walled and excystment takes place in two stages (Crump, 1950). The amoebae were grown on non-nutrient agar (2·5 %, w/v, agar), 0·5 % (w/v) NaCl; pH 6·8–7·0; plates supplied with a young culture (3 days old) of *Aerobacter* sp. grown on nutrient agar slopes (Strain 1912; Singh, 1941*a*). Ten-day-old cysts were collected and washed three times by suspension and centrifugation in sterile distilled water at 500 rev./min. for 3 min., in order to get rid of most of the bacteria. They were then treated with a mixture of penicillin (200 units/ml.) and streptomycin (1000 units/ml.) for 48 hr. at 25° to kill bacteria, centrifuged, and washed with distilled water. The cysts were next treated with 0·5 % (w/v) emetine hydrochloride or HCl (1·0–1·5 %, w/v) for 24 hr. to destroy active amoebae or partially formed cysts. After washing, these sterile cysts were kept in 0·85 % (w/v) NaCl solution at 4° and were used for excystment experiments during 4 weeks, when there was hardly any loss in viability. The freedom of these cysts from bacteria was tested on nutrient agar slopes or in nutrient broth, and their viability by inoculating them in the presence of living *Aerobacter* sp. on non-nutrient agar. The sterile cysts excysted readily in the presence of living bacteria on the non-nutrient agar, although on non-nutrient agar alone there was hardly any excystment.

Method of studying excystment

Petri dishes, each containing a flat-bottom cavity slide placed on a piece of filter paper, were sterilized (160° for 1 hr.). A very tiny drop (*c.* 0·001 ml.) of cyst suspension, containing 50–125 cysts, was transferred to the centre of the cavity slide by a finely drawn capillary pipette. A small drop of the test fluid (*c.* 0·005–0·01 ml.) was then added and the filter paper was moistened with sterile distilled water to prevent evaporation of the liquid in the cavity slide. These Petri dishes were then incubated at 24–25° for 18–24 hr. After this period, the cavity of the slide was sealed with a sterile coverslip, the slide inverted carefully and examined under the low power of a microscope. The fluid containing the cysts remained as a drop and did not spread out. A cyst was considered excysted only when an amoeba escaped from it and was found to be moving freely in the surrounding medium. The percentage excystment was calculated from the count of the amoebae and the unexcysted cysts. As there was no food supply for the excysted amoebae, they remained viable for 72–96 hr. and then died or tried to encyst. To ascertain whether sterile conditions had prevailed during the experiment, the fluid from the cavity slides was drawn out at the end of the experiment and was inoculated into nutrient broth or on nutrient agar.

Preparation of the aqueous extracts of bacteria

(1) *Aerobacter* sp., *Escherichia coli* and *E. coli* strain RCI (phage-resistant strain of *E. coli*; Gupta, 1957) were grown in a liquid defined medium (NH_4Cl, 1 g.; $MgSO_4.7H_2O$, 0·1 g.; KH_2PO_4, 1·5 g.; Na_2HPO_4, 3·5 g.; lactic acid, 9·0 g.; distilled water, 1000 ml.; pH 6·8–7·0) at 37° for 17 hr. The organisms were washed thrice with distilled water by centrifugation. They were then killed by suspending them in ice-cold acetone for 30 min., washed once with acetone and dried in a vacuum desiccator. The dried organisms were ground with sterile glass dust and distilled water in a mortar, and the water-soluble portion collected by centrifugation. This aqueous extract was sterilized at 15 lb./sq.in. pressure for 15 min. and had a pH value of about 6·8–7·0. The bacterial residue was washed three times and suspended in distilled water.

(2) The washed *Aerobacter* sp. (17 hr. old) was suspended in distilled water, heated at 100° for 20 min. in a water bath (Freeland & Gale, 1947) and centrifuged. The supernatant liquid was at pH 6·5. The organisms were washed three times and suspended in distilled water.

RESULTS

Effect of aqueous extracts of bacteria on excystment

The excystment properties of the aqueous extract and of the bacterial residue suspension of the *Aerobacter* sp., *Escherichia coli*, *E. coli* strain RCI, distilled water and normal saline were tested in duplicate; the results are given in

Table 1. *The effect of various preparations on excystment*

	Duration of storage at 4° (days)				
	1	3	5	7	14
Preparation	Degree of excystment (%)*				
Extract of:					
Aerobacter sp.	96	92	95	85	87
Escherichia coli	93	90	—	83	76
E. coli strain RCI	96	—	93	—	—
Bacterial residue suspended in distilled water	Nil	Nil	Nil	Nil	Nil
Distilled water (control)	Nil	1·5	Nil	Nil	Nil
Normal saline	Nil	Nil	2·6	Nil	Nil

* Means of duplicate set.

Table 1. The variation in the % excystment of duplicate sets was very little. The fact that the aqueous extract showed marked ability to induce excystment while the bacterial residue suspension was unable to do so, indicated that the bacterial products which induce excystment are water soluble. Storage of the extracts up to a period of 14 days at 4° or autoclaving at 15 lb./sq.in. for 20 min. had practically no adverse effect on excystment.

It is known (Singh, 1946) that cysts produced on different occasions under

similar cultural conditions may vary in their % viability. Therefore, it is important to know the % viability of a batch of cysts before using them in excystment experiments. The ability of the extracts of *Aerobacter* sp. and of *Escherichia coli* to cause a high % excystment provided a useful method of finding out the % viability in a particular batch of cysts.

Effect of pH *value of a bacterial extract on excystment*

The extract of acetone-killed *Aerobacter* sp. (pH. 6·8) was adjusted to different pH values between 2 and 8 by adding HCl or NaOH. There was no excystment at pH 2–3; pH 6–8 was found to be most favourable range for the excystment of *Schizopyrenus russelli.*

Characterization of excystment-causing factors from the water-soluble extract of Aerobacter *sp.*

The water-soluble extract (W) of *Aerobacter* sp. which showed 93 % excystment, obtained from organisms killed by acetone, was evaporated to dryness below 50°, under reduced pressure. It was extracted with 80 % (v/v) ethanol in water and centrifuged. The insoluble residue (R) when dried and dissolved in distilled water, showed a greatly decreased ability to cause excystment. The ethanolic solution (E) was evaporated to dryness and the residue dissolved in distilled water; it was very effective in causing excystment. This solution showed a strong positive ninhydrin reaction, a positive test for sugars (aniline hydrogen phthalate) and a positive test for organic phosphate. This material was fractionated by paper partition chromatography. The solution was applied to a sheet of Whatman No. 1 filter paper as a streak and the paper developed with butanol + acetic acid + water (4:1:5) as the solvent system by the descending technique. The solvent was allowed to travel to the edge of the paper, the paper dried and a narrow segment of the chromatogram cut from one side and sprayed with ninhydrin for the location of the amino acids. Nine distinct ninhydrin-positive spots were visible. The chromatogram was then divided into twenty bands, ten corresponding to the ninhydrin-positive bands and ten other bands. These bands were excised, eluted with water and the eluates autoclaved and tested for their excystment properties. Six of these fractions showed excystment activity; five of these corresponded with the five slower-moving ninhydrin-positive bands. The sixth fraction (R_F 0·53–0·63) with a ninhydrin-negative zone; this fraction has not yet been identified.

Paper chromatography of the aqueous extract (W) was repeated with the same solvent system. To obtain better resolution of the amino acids, the chromatogram was allowed to run 30 hr. and a segment of the paper developed with ninhydrin as before. Again nine strong and two weak ninhydrin-positive spots were obtained. The bands corresponding to the nine distinct ninhydrin-positive spots were marked on the chromatogram, the bands excised, eluted with water and the eluates tested for the excystment activity. Table 2 gives a summary of the results, the R_i value of the fractions and the corresponding % excystment obtained. It is clear from Table 2 that only the six slow-moving fractions showed excystment activity; of these, fractions one and five

were most active. The inability of the other fractions to cause excystment may have been due to a low concentration of these amino acids. The amino acids identified in these fractions by two-dimensional chromatography were cystine, lysine, arginine, histidine, aspartic acid or glycine, glutamic acid, alanine, tyrosine, valine, isoleucine or phenylalanine (Table 2). The solvent systems used were butanol + acetic acid + water (4:1:5) and phenol + water + ammonia (80:20 with 3% ammonia). Further work to characterize the excystment-causing material insoluble in 80% (v/v) ethanol in water and the determination of the relative concentration of amino acids is in progress.

Table 2. *Excystment caused by amino acids isolated from the water-soluble extract of* Aerobacter *sp. by paper chromatography*

Fractions	Ri* in BuOH:AcOH:H_2O 4 1 5	pH value	Identified amino acid	Degree of excystment† (%)
1	0·140	6·0	Cystine	67
2	0·187	6·5	Lysine	39
3	0·238	6·5	Arginine/histidine	40
4	0·355	6·0	Aspartic acid or glycine	19
5	0·410	6·0	Glutamic acid	66
6	0·477	6·0	Alanine	7
7	0·618	6·0	Tyrosine	Nil
8	0·79	6·5	Valine	Nil
9	1·00	6·0	Isoleucine or phenylalanine	Nil

* R_i refers to the distance travelled by individual fractions from the origin as compared to that of isoleucine taken as 1.

† Mean of duplicate sets.

The finding that the bacterial products of the *Aerobacter* sp. which caused excystment were water soluble and that part of this activity was due to amino acids, led to the testing of various pure amino acids for their excystment inducing activity. Certain sugars, purines, pyrimidines, nucleosides, nucleotides, and vitamins B_1, B_2, B_6 were also examined.

Effects of various amino acids on excystment. The excystment inducing activity of twenty-three amino acids dissolved in distilled water and autoclaved was tested (Table 3). Some of them induced good excystment, while others induced poor or no excystment. Since the low pH value of the aqueous extract of *Aerobacter* sp. was a limiting factor and pH values between 6 and 8 were most favourable for excystment, some of the amino acids were tested at different pH values. Solutions of DL-histidine dihydrochloride, DL-lysine dihydrochloride, L-cysteic acid, L-histidine and glutathione, which gave no excystment at pH 1·0–3·0 gave good excystment at pH 7. There were, however, some amino acids which caused poor or no excystment even in the favourable pH range of 5·0–7·0 (Table 3).

To study the effect of the concentration of amino acids on excystment, some of the amino acids were tested at different concentrations (pH 6–7); the results are recorded in Table 4. It was found that a definite concentration

was necessary to give good excystment. There was a certain degree of variation in % excystment when cysts from different batches were tested with the same amino acids. Therefore, to get comparative % excystment, different substances should be tested with the same batch of cysts.

Table 3. *The effect of various amino acids on excystment*

Amino acid	Concentration	pH	Degree of excystment* (%)
DL-Valine	2·0 % (w/v)	6·0	68
DL-Serine	2·0 % (w/v)	6·8	69
DL-Methionine	2·0 % (w/v)	6·0	52
Glycine	2·0 % (w/v)	6·0	45
DL-Histidine dihydrochloride	2·0 % (w/v)	1·0	Nil
DL-Threonine	2·0 % (w/v)	5·0	49
DL-Proline	2·0 % (w/v)	7·0	47
DL-Isoleucine	2·0 % (w/v)	6·5	53
DL-Lysine dihydrochloride	2·0 % (w/v)	1·0	Nil
DL-β-Phenylalanine	2·0 % (w/v)	6·0	27
L-Asparagine	2·0 % (w/v)	5·0	Nil
L-Glutamic acid	2·0 % (w/v)	6·0	61
DL-Aspartic acid	2·0 % (w/v)	3·0	Nil
DL-Leucine	2·0 % (w/v)	6·5	7
DL-α-Alanine	2·0 % (w/v)	6·0	15
L-Hydroxyproline	5·0 % (w/v)	7·2	76
L-Cysteic acid	5·0 % (w/v)	1·0	Nil
L-Histidine	5·0 % (w/v)	1·0	Nil
L-Arginine	5·0 % (w/v)	7·0	25
DL-Ornithine HCl	5·0 % (w/v)	6·0	2
Glutathione	5·0 % (w/v)	3·0	Nil
DL-Alanyl-D-asparagine	5·0 % (w/v)	6·0	9
Sarcosine	5·0 % (w/v)	5·0	Nil

* Mean of duplicate sets.

Table 4. *The effect of the concentration of amino acids on excystment*

	Dilutions			
	2·0 % (w/v)	1·0 % (w/v)	0·2 % (w/v)	0·1 % (w/v)
	Degree of excystment* (%)			
L-Glutamic acid	95	88	—	2
DL-Histidine	88	83	13	Nil
DL-Isoleucine	—	48	57	20
L-Cysteic acid	67	75	25	9
DL-Lysine	—	65	10	3

* Mean of duplicate sets

Effect of sugars on excystment. 1·0 and 0·5 % (w/v) aqueous solutions of arabinose, maltose, raffinose, glucose, lactose, ribose and glucosamine were sterilized by autoclaving (15 lb./sq.in. for 20 min.) and tested for their excystment properties. There was no excystment in the presence of these compounds.

Effect of purines, pyrimidines, nucleosides, nucleotides and some organic phosphates on excystment. As certain purines, pyrimidines, nucleosides and nucleotides or organic phosphates might be present in the free state in the metabolic pool in the bacteria, 1·0 % (w/v) solutions of uracil, uridine, cytidine, adenosine, uridylic acid, guanylic acid, yeast and muscle adenylic acids, and 0·5 % (w/v) solutions of adenosine triphosphate, β-glycerophosphate, fructose-6-phosphate, glucose-1-phosphate, potassium metaphosphate and 0·2 % (w/v) solutions of cytosine, hypoxanthine and adenine were tested for their ability to induce excystment. Only muscle adenylic acid, adenosine triphosphate and fructose-6-phosphate induced excystment of 35, 65 and 42 % respectively; the other compounds were ineffective. Muscle adenylic acid was tested at 2·0, 1·0, 0·2 and 0·1 % (w/v) concentrations and the percentage excystment of these was 42, 35, 9 and 3 %, respectively, showing that higher concentrations caused better excystment.

Effect of vitamins on excystment. Samples of vitamins B_1, B_2 and B_6 were dissolved in distilled water, autoclaved and tested for excystment properties. Only vitamin B_1 at a dilution of 1·0 % (w/v) induced excystment.

Excystment of Schizopyrenus russelli *cysts at different intervals in the presence of excystment inducing agents.* 1·0 % (w/v) solution of L-glutamic acid, L-cysteic acid and aqueous extract of acetone-killed *Aerobacter* sp. at pH 7·0 were used in this experiment, keeping distilled water as control. In the presence of the bacterial extract the excystment was somewhat more rapid in the first few hours, and at the end of 24 hr. all the viable cysts had excysted. The excysted amoebae remained viable in amino acids during 96 hr. without multiplying or encysting.

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